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Mutational Analysis of Early Region 4 of Bovine Adenovirus Type 3[1]

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The primary objective of characterizing bovine adenovirus type 3 (BAV3) in greater detail is to develop it as a vector for gene therapy and vaccination of humans and animals. A series of BAV3 early region 4 (E4) deletion-mutant viruses, containing deletions in individual E4 open reading frames (Orf) or combinations of Orfs, were generated by transfecting primary fetal bovine retinal cells with E4-modified genomic DNA. Each of these mutants was further analyzed for growth kinetics, viral DNA accumulation, and early/late protein synthesis. Mutant viruses carrying deletions in Orf1, Orf2, Orf3, or Orf4 showed growth characteristics similar to those of the E3-deleted BAV3 (BAV302). DNA accumulation and early/late protein synthesis were also indistinguishable from those of BAV302. However, mutant viruses carrying a deletion in Orf5, Orfs 1–3 (BAV429), or Orfs 3–5 (BAV430) were modestly compromised in their ability to grow in bovine cells and express early/late proteins. E4 mutants containing larger deletions, Orfs 1–3 (BAV429) and Orfs 3–5 (BAV430), were further tested in a cotton rat model. Both mutants replicated as efficiently as BAV3 or BAV302 in the lungs of cotton rats. BAV3-specific IgA and IgG responses were detected in serum and at the mucosal surfaces in cotton rats inoculated with mutant viruses. *In vitro* and *in vivo* characterization of these E4 mutants suggests that none of the individual E4 Orfs are essential for viral replication. Moreover, successful deletion of a 1.5-kb fragment in the BAV3 E4 region increased the available insertion capacity of replication-competent BAV3 vector (E3–E4 deleted) to ~4.5 kb and that of replication-defective BAV3 vector (E1a–E3–E4 deleted) to ~5.0 kb. This is extremely useful for the construction of BAV3 vectors that express multiple genes and/or regulatory elements for gene therapy and vaccination.

INTRODUCTION

Adenovirus gene expression can be divided into early and late phases, which are separated by the onset of viral DNA replication (Persson and Philipson, 1982). As in human adenoviruses (HAV), bovine adenovirus type 3 (BAV3) contains early genes E1, E2, E3, and E4 that are transcribed from individual promoters and are located on both strands (Baxi *et al.,* 1999; Idamakanti *et al.,* 1999; Reddy *et al.,* 1999a). In contrast, late genes are generated by alternative splicing of a single polycistronic mRNA transcribed from a single promoter, the major late promoter. In BAV3, late mRNAs can be divided into seven families (L1 to L7) compared to five in HAVs (Reddy *et al.,* 1998). In general, the early genes function in the regulation of viral transcription, transformation, and viral DNA replication, while the late genes code for structural proteins.

The adenovirus early region 4 (E4) of HAV constitutes ~10% of the viral genome and genetic analysis has demonstrated that some of the E4 products are essential for virus infection (reviewed by Leppard, 1997). A complex set of phenotypes has been demonstrated by deletion analysis of E4, showing that E4 products play vital roles in virus infection. E4 proteins are involved at several levels of regulation of cellular and viral gene expression, viral DNA replication, late viral assembly, E2 expression, and adeno-associated virus helper function (Halbert *et al.,* 1985; Huang and Hearing, 1989; Richardson and Westphal, 1981; Weinberg and Ketner, 1986). Several sets of differentially spliced mRNAs are generated from the E4 region during viral infection (Dix and Leppard, 1993; Freyer *et al.,* 1984; Tigges and Raskas, 1984; Virtanen *et al.,* 1984). Of the possible seven open reading frames (Orf) encoded by the E4 region of HAV, Orf3 or Orf6 is absolutely essential for viral growth in tissue culture (Bridge and Ketner, 1989; Huang and Hearing, 1989; Ketner *et al.,* 1989). Both proteins increase viral late protein production by facilitating the accumulation of mRNAs at a posttranscriptional level. E4 Orf6 forms a physical and functional complex with E1B-55kDa (Bridge and Ketner, 1990; Rubenwolf *et al.,* 1997) and helps it to target the 55k protein to the nucleus (Ornelles and Shenk, 1991). It has also been suggested that this E4 Orf6–55k complex shuttles between the two cellular compartments and serves as a nucleocytoplasmic transporter of viral mRNAs (Dobbelstein *et al.,* 1997; Weigel and Dobbelstein, 2000) and also inhibits the export of most cellular mRNAs (Babiss *et al.,* 1985). E4 Orf6 also has a direct effect on transactivation of p53 by forming a tertiary complex between p53, E4 Orf6, and 55k, leading to degradation of p53 (Boivin *et al.,* 1999; Boyer and

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Ketner, 2000; Cathomen and Weitzman, 2000). The E4 Orf6 can also block p53-dependent apoptosis (Moore *et al.,* 1996) and can cooperate with E1A to transform primary rodent cells (Moore *et al.,* 1996; Nevels *et al.,* 1997). E4 Orf3 appears to relieve the E1B inactivation of p53 independently and plays a role in relocating sites of replication and transcription in the nucleus (Leppard and Everett, 1999).

BAV3, a representative of subgroup I of BAVs, has been shown to replicate in cattle, producing mild or no clinical symptoms (Darbyshire *et al.,* 1965). The complete genome of BAV3 has been sequenced (Baxi *et al.,* 1998; Lee *et al.,* 1998; Reddy *et al.,* 1998) and E1, E3, and E4 regions (Baxi *et al.,* 1999; Idamakanti *et al.,* 1999; Reddy *et al.,* 1999a) have been characterized at the transcriptional level. It has also been demonstrated that the E3 region of BAV3 is not essential for viral replication (Zakhartchouk *et al.,* 1998) and vaccine antigens expressed in the E3 region can induce protective mucosal and systemic immune responses (Baxi *et al.,* 2000; Zakhartchouk *et al.,* 1999). The BAV3 E1A region is essential for viral replication and E1A-deleted virus can be generated only in bovine cell lines expressing HAV5 E1 (Reddy *et al.,* 1999b). We are characterizing BAV3 with the aim of developing it as the vector of choice for human gene therapy and vaccination in human and animals.

Early region 4 of BAV3 is located at the extreme right end of the BAV3 genome from map unit 98.6 to 89.9 (Lee *et al.,* 1998). The entire BAV3 E4 transcriptional unit is 3004 bp in size and transcribes seven distinct 5′–3′ coterminal mRNAs, which have potential to encode five different polypeptides (Baxi *et al.,* 1999). Although proteins encoded by Orf3 and Orf5 of BAV3 E4 show partial homology to the 34-kDa protein of HAV2 E4 region and its homologs, the conserved motif HCHXXPGSLQ present in the 34-kDa protein of HAV2 (Virtanen *et al.,* 1984), the 30.8-kDa protein of OAV287 (Vrati *et al.,* 1996), and the a/b Orf of MAV1 (Kring *et al.,* 1992) is present only in BAV3 E4 Orf5. Since no homologs were observed in other adenoviruses, Orfs 1, 2, and 4 appear to be unique to BAV3.

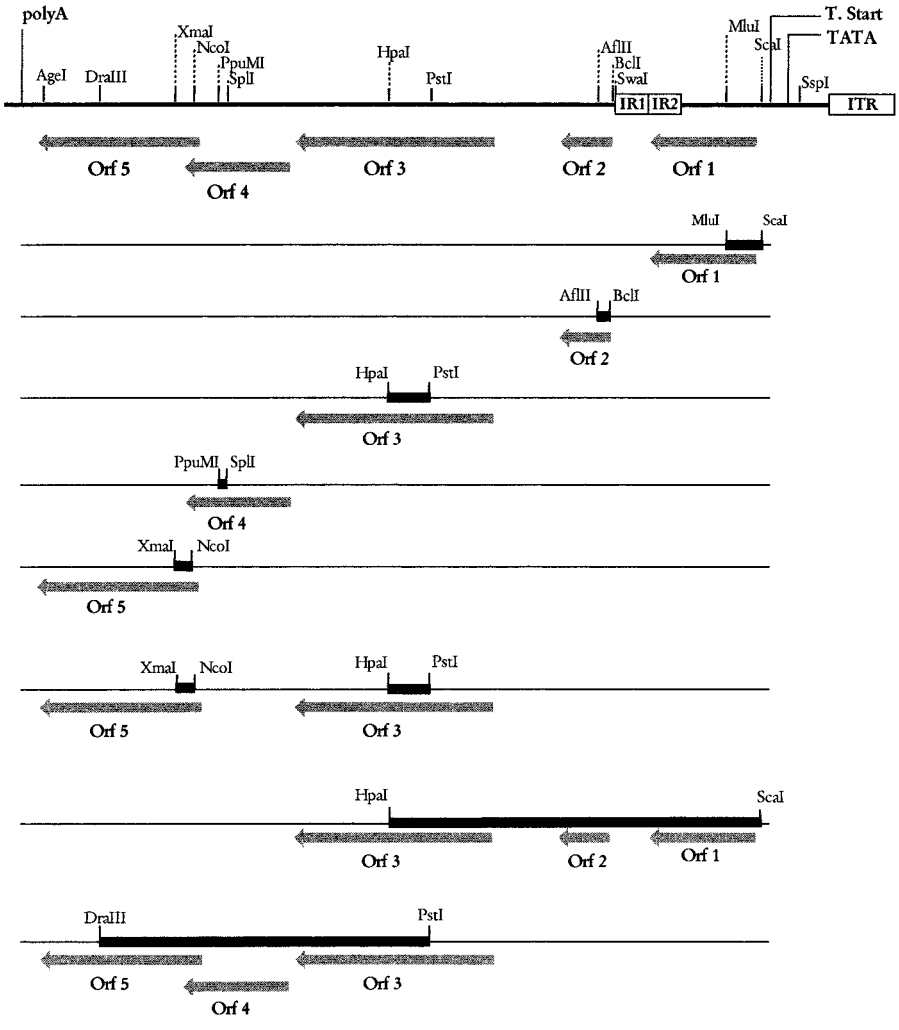
In this report, we describe the construction and characterization of BAV3 E4 deletion mutants, both *in vitro* and *in vivo,* with the aim of further analyzing the BAV3 E4 region in detail and increasing the insertion capacity of BAV3 vector by deleting the nonessential E4 region.

RESULTS

Construction and analysis of E4 mutants

Studies done in HAV E4 suggest that multiple gene products encoded by E4 share redundant properties. In order to study functions for gene products encoded by BAV3 E4, we constructed a series of E4 mutant viruses. Initially, each of the Orfs were serially deleted, followed by larger deletions by combining the individual E4 Orfs (Fig. 1). All the mutant viruses were generated by transfecting fetal bovine retina cells (FBRC) with individual full-length plasmid DNA containing the various E4 deletions. Mutant BAV419 contains a deletion of 143 bp that disrupts Orf1 with the insertion of an *Eco*RV linker. Mutant BAV401 contains a deletion of 58 bp that disrupts Orf2 with the insertion of a *Srf*I linker. Mutant BAV427 contains a deletion of 172 bp, disrupting Orf3, and has a *Srf*I linker inserted. BAV411 and BAV412 contain deletions of 38 and 78 bp, respectively, disrupting the Orf4 and Orf5 genes, and have a *Srf*I linker. In order to disrupt Orf3 and Orf5 simultaneously mutant BAV415, which contains a total deletion of 250 bp, was generated. Two larger deletions were created in the E4 region of BAV3 in order to identify the regions essential for viral replication. Mutant BAV429 contains a deletion of 1501 bp and disrupts Orfs 1, 2, and 3 and contains a *Srf*I linker. BAV430 mutant disrupts Orfs 3, 4, and 5 and contains a deletion of 1342 bp and a *Srf*I linker. The various deletions completely disrupt the Orfs with the exception of mutant BAV411, in which Orf4 contains internal deletion. However, mutant BAV430 contains a deletion from Orf3 to 5 that completely disrupts Orf4. Surprisingly, we were unable to generate BAV3 mutant virus completely devoid of the E4 region. All the mutant viruses constructed have an E3 deletion (1.245 kb; Zakhartchouk *et al.,* 1998) in the background.

In order to confirm the presence of the specific mutations/deletions, two different approaches were used: first the viral DNA was isolated and digested with unique restriction enzymes. Viral DNA from BAV419 was digested with restriction enzyme *Eco*RV. Two bands were observed upon digestion of BAV302 (E3-deleted virus) viral DNA with *Eco*RV, since only one *Eco*RV site is present in BAV302 (Fig. 2). However, BAV419 has an additional *Eco*RV site because of the insertion of the *Eco*RV linker, hence we observe three bands upon digestion of BAV419 DNA with *Eco*RV (Fig. 2). Viral DNA from BAV401, BAV427, BAV411, BAV412, BAV415, BAV429, and BAV430 was digested with *Srf*I. All these mutant viruses show an additional band compared to BAV302 (only one *Srf*I site in BAV302 DNA; Fig. 2), because of the insertion of the *Srf*I linker in all of these mutant viral genomes. Second, the identity of the mutant was confirmed by PCR analysis. Primers were generated from the flanking regions of the deletions (Table 1). Mutant viral DNAs were PCR amplified and observed for a shift in the size of their products, generated due to deletions in the mutants, compared to BAV3 DNA. The results of the PCR analyses are summarized in Fig. 3 and Table 1. Compared to the BAV3 control, all the deletion mutants yielded the expected smaller amplification products.



Virus	Deletion size	Linker	Orf disrupted
BAV419	143bp	EcoRV	Orf1
BAV401	58bp	SrfI	Orf2
BAV427	172bp	SrfI	Orf3
BAV411	38bp	SrfI	Orf4
BAV412	78bp	SrfI	Orf5
BAV415	250bp	SrfI	Orf3&5
BAV429	1501bp	SrfI	Orf1-3
BAV430	1342bp	SrfI	Orf3-5

FIG. 1. E4 transcription map and mutant viruses. The right end of BAV3 genome (30,833–34,446 nucleotides, as determined by Lee *et al.,* 1998) is represented at the top. Some of the unique features of the E4 region, such as internal repeats (IR1 and IR2), TATA box, E4 transcription start site (T. Start), right inverted terminal repeat (ITR), and poly(A), are also illustrated. The predicted five open reading frames are indicated by shaded arrows showing the direction of transcription. Relevant restriction endonuclease cleavage sites that are used in the construction of E4 deletion mutants are also marked. Each of E4 deletion mutants described in the text are illustrated individually, with the solid black bars corresponding to the deletions created in each of E4 mutants.

In vitro characterization of BAV3 E4 mutants

Virus replication in vitro. In order to determine whether a single Orf deletion or larger deletions had a noticeable effect on the ability of BAV3 to replicate *in vitro,* growth kinetics of the individual E4 mutant viruses were analyzed in MDBK cells (Fig. 4). Cultures of MDBK cells were infected with mutant viruses and cells harvested at 6, 12, 24, 36, 48, and 72 h postinfection. Virus from each sample was released by freeze–thawing and titered on MDBK cells. Mutant viruses with deletions in Orf1, Orf2, Orf3, and Orf4 grew with comparable efficiencies compared to BAV302 (E3-deleted) virus. However, the mutant viruses that have deletions in Orf5 and Orfs 3 and 5 and mutant viruses that have a larger deletion, Orf1–3 and Orf3–5, showed slower growth characteristics compared to BAV302. These mutant viruses (BAV412, BAV415, BAV429, and BAV430) produced 2–3 logs less virus compared to BAV302 at 24, 36, and 48 h postinfection. However, the final yields of these viruses were within 1 log compared to that of BAV302.

Viral DNA replication. Viral DNA replication was assayed at various times postinfection of MDBK cells with the BAV302 or mutant viruses. Viral DNA was isolated at 24, 36, and 48 h after infection, and viral DNA accumulation was measured by dot blot analysis using ^{32}P-labeled adenovirus genomic DNA as a probe (Fig. 5). The levels of viral DNA at 24 h after infection were comparable among various mutant viruses to that observed in the BAV302 virus. However, at 36 and 48 h postinfection, a lag in the onset of viral DNA replication was observed specifically in the viruses that carry larger deletions. Based on densitometric analysis ($n = 3$) there was a significant two- to three-fold reduction of viral DNA accumulation compared to BAV302 at 36 and 48 h postin-

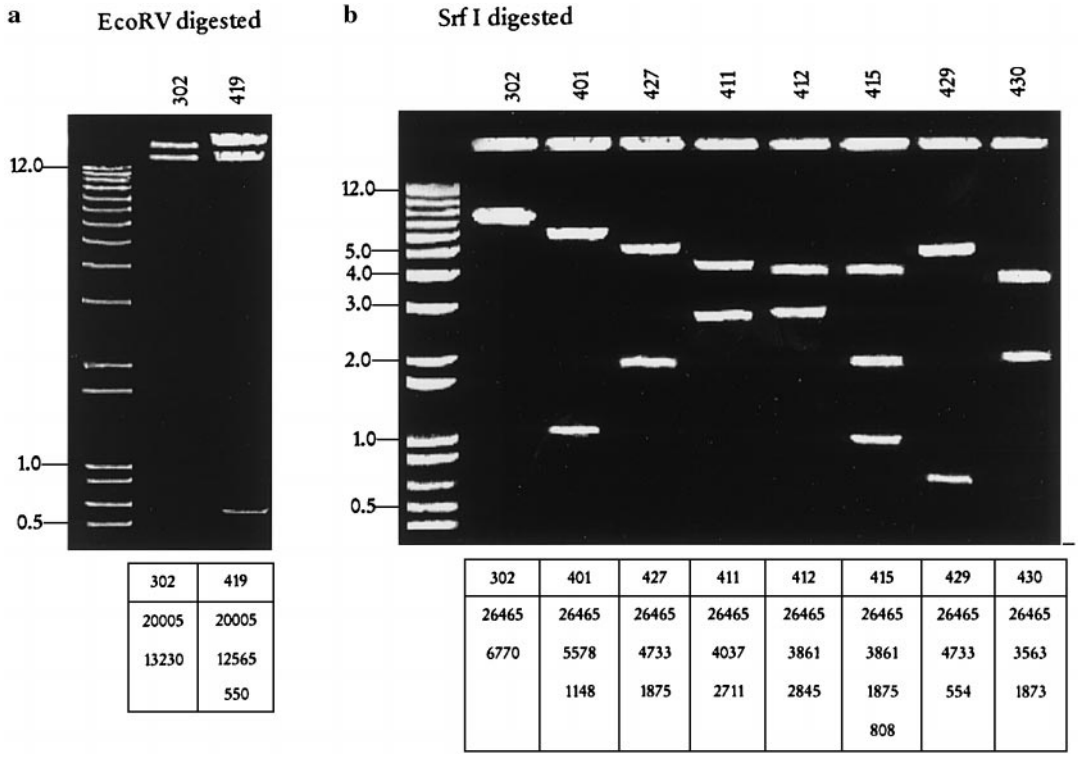


FIG. 2. Restriction enzyme analysis of the mutant viruses. MDBK cells were infected with various mutants and viral DNA was extracted for each of the mutant viruses. The DNA from mutant BAV419 was digested with *Eco*RV and DNA from mutant viruses BAV401, BAV427, BAV411, BAV412, BAV415, BAV429, and BAV430 was digested with *Srf*I. BAV302 DNA was digested with *Eco*RV and *Srf*I as a control. A 1 kb DNA ladder (lane M) was used for sizing the DNA fragments. Expected DNA fragment sizes generated upon digestion with unique restriction enzymes are shown below each of the individual mutant viruses.

fection but not at 24 h. The mutant viruses that have only one Orf disrupted accumulated viral DNA with similar kinetics compared to the BAV302 virus.

Viral early and late protein synthesis. In order to study the effect of the deletion on viral protein synthesis, MDBK cells were infected with BAV302 or mutant viruses and labeled with [^{35}S]-methionine. Proteins from cellular lysates were immunoprecipitated with rabbit polyclonal antibodies specific for BAV3 early region 1B small (E1B^{small}), DNA binding protein (DBP), or fiber and ana-

TABLE 1

PCR Analysis of the BAV3 E4 Recombinants

Virus	Primers	Orf deleted	Expected size in BAV3 (bp)	Expected size in recombinants (bp)
BAV419	5′TTCACTTGCGGCAGCTC3′ 5′TCAACGATGAGGACCAGCTGA3′	Orf1	456	311
BAV402	5′ATTCTTGGCACAGTCTTCG3′ 5′CAATCCAGTGGTCTCTC3′	Orf2	392	334
BAV427	5′CAATTCCGACAGCTGAG3′ 5′CATCGACTAGCAGACAC3′	Orf3	590	418
BAV411	5′TATGGTGGACCAAGTGC3′ 5′CAACTAGTCAAGTGAGCGTCCT3′	Orf4	435	397
BAV412	5′CACTATGATTCACGGCCCATTCC3′ 5′GACAAGTCATACCGGTC3′	Orf5	648	570
BAV415	5′CAATTCCGACAGCTGAG3′ 5′GACAAGTCATACCGGTC3′	Orf3 and 5	1835	1585
BAV429	5′CATTGTTCCGTGTTCTGC3′ 5′CATCGACTAGCAGACAC3′	Orf1–3	1892	391
BAV430	5′CAATTCCGACAGCTGAG3′ 5′GACAAGTCATACCGGTC3′	Orf3–5	1835	493

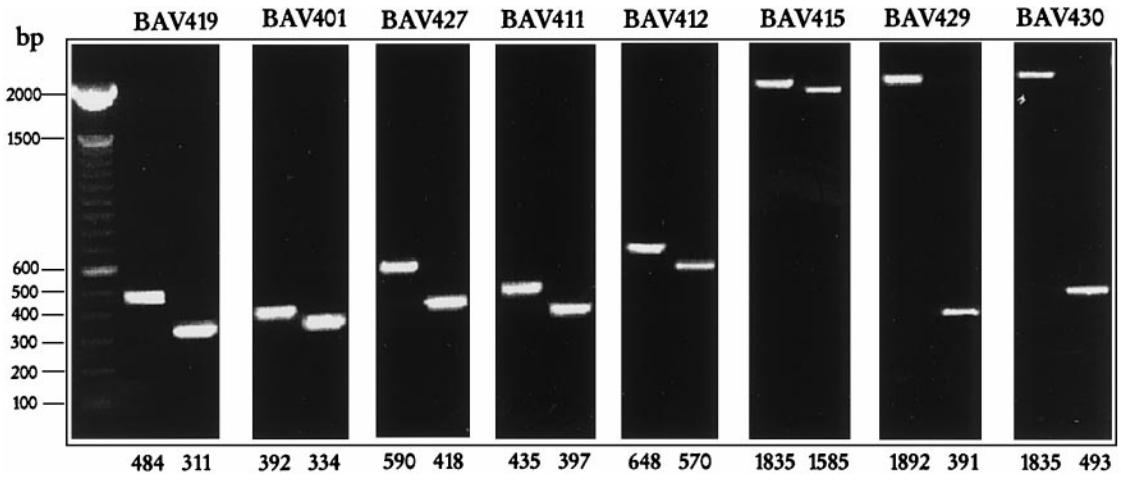


FIG. 3. Genomic PCR of the viral mutants. PCR-amplified products generated by primers flanking the individual E4 deletions are shown in comparison to PCR products generated by amplification of the similar region from BAV3 DNA. The expected sizes of amplified products generated by PCR from BAV3 and mutant viruses are also shown at the bottom. Molecular size markers are indicated on the left.

lyzed by SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 6). Densitometric analysis of early protein production by BAV302 and mutant viruses suggests that mutant viruses expressed similar or higher amounts of $E1B^{small}$ and DBP protein compared to BAV302 (Figs. 6a and 6b). However, the amount of viral late protein synthesis as observed by fiber production was reduced in all the mutants compared to BAV302, with the exception of BAV430 (Fig. 6c).

In vivo characterization of BAV3 E4 mutants

Virus replication in respiratory tract of cotton rats. In order to determine whether E4 deletions have any effect on kinetics of virus replication and clearance from the lungs of inoculated cotton rats, titers of infectious virus recovered from the lungs of animals inoculated with either BAV429 or BAV430 were compared with that of BAV302 and wild-type BAV3. Following intranasal inoculation of cotton rats with 10^7 pfu of recombinant viruses, there was a decline in virus titers in all four groups at day 2 p.i., followed by 1 log increase in virus titers on day 3 p.i. (Fig. 7). This increase in the amount of virus titer might be a result of active virus replication. There was a

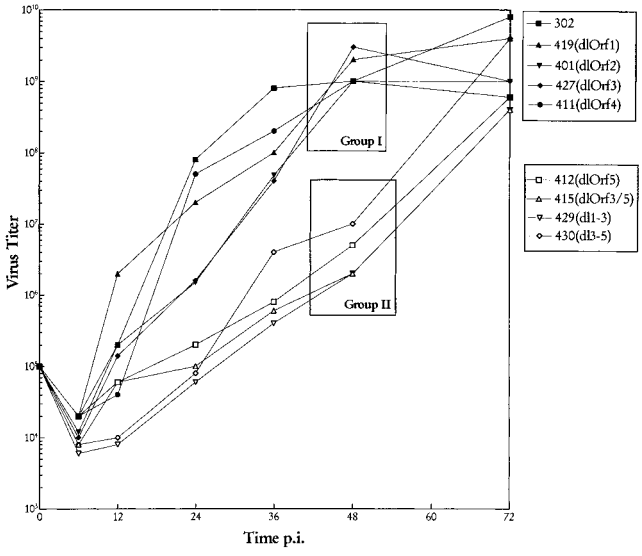


FIG. 4. Growth kinetics of the E4 mutant viruses. Confluent monolayers of MDBK cells were infected at m.o.i. of 0.1 with BAV302 (E3 deleted) and mutant viruses. Infected MDBK cells were harvested at the indicated times postinfection, and the amounts of virus in cell lysates were determined by plaque assay and represented as plaque-forming units/ml.

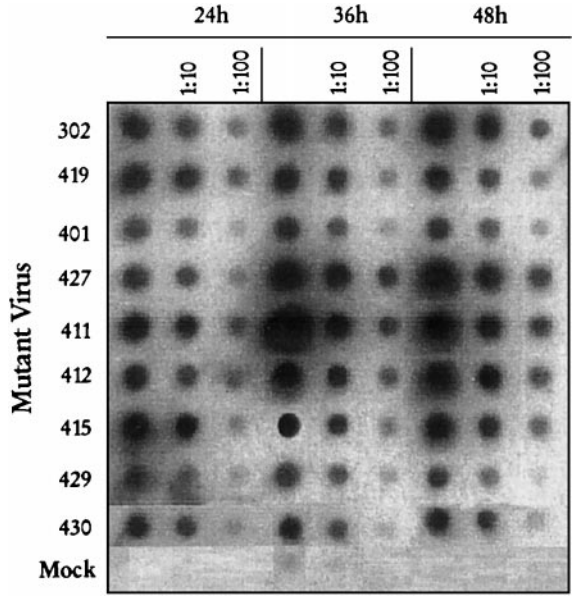


FIG. 5. DNA replication assay. Total viral DNA was isolated from MDBK cells that were infected with BAV302 and mutant viruses at m.o.i. of 10. The DNA was isolated at various time points, denatured, neutralized, and diluted as indicated and transferred onto the nitrocellulose membrane by using the dot-blot apparatus. The blot was hybridized with ^{32}P-labeled BAV3 genomic probe. The hybridized DNA was visualized by autoradiograph.

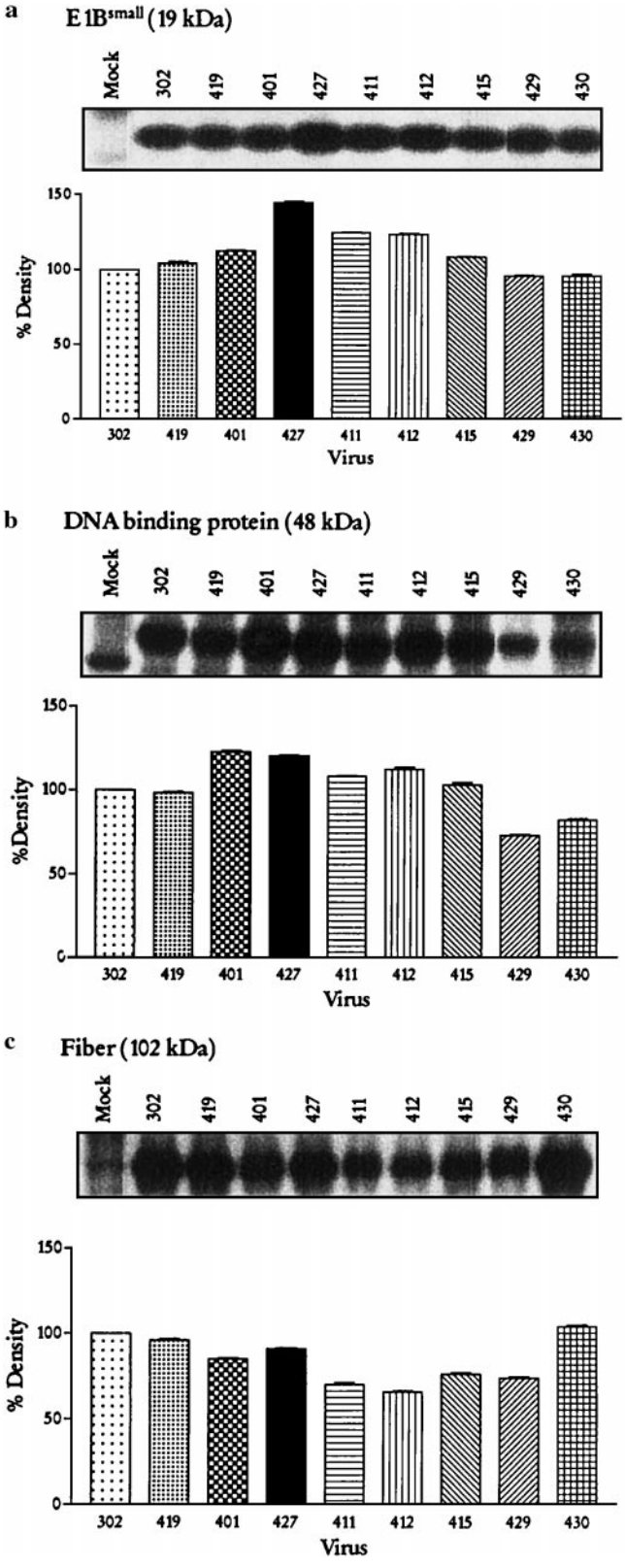


FIG. 6. Viral early and late protein synthesis. Confluent monolayers of MDBK cells were either mock infected or infected with BAV302 and various mutant viruses at m.o.i. of 10 and labeled with [^{35}S]methionine. At 24 h postinfection, proteins from lysates of radiolabeled cells were immunoprecipitated with (a) E1B^{small}, (b) DBP, or (c) fiber polyclonal antibody and analyzed by 10% SDS–PAGE. Specific bands of 19, 48, and 102 kDa were observed for E1B^{small}, DBP, and fiber proteins, respectively, under reducing conditions. Results of densitometric scanning of the E1B^{small}, DBP, or fiber immunoprecipitations are also shown below each immunoprecipitation as mean values for triplicate samples ±SD. The expression of proteins in BAV302 is taken as 100%, with other results expressed relative to it.

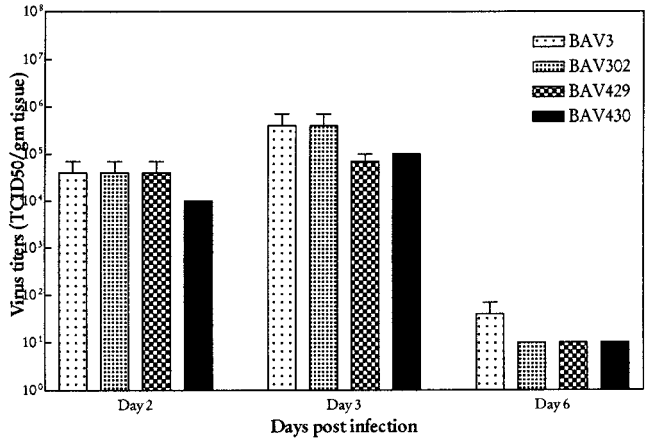


FIG. 7. Virus replication in the lungs of cotton rats. Animals were inoculated intranasally with 10^7 pfu of BAV3, BAV302, BAV429, or BAV430 and lungs were collected at days 2, 3, and 6. Each time point represents mean virus titers for three animals ± SD.

4–5 log decline in virus titer on day 6 p.i. Over the 6-day period, the amounts of infectious virus shed by both mutant viruses were similar to those recovered from lungs of BAV302- and BAV3-inoculated cotton rats (Fig. 7).

Immune response against mutant viruses in cotton rats. In order to study the immune response generated by deletion mutants, cotton rats were inoculated intranasally at 0 and 3 weeks with BAV3, BAV302, BAV429, and BAV430 viruses. The serum samples were collected at 3 and 6 weeks, and nasal and lung washes were collected at 6 weeks after primary infection. The sera were tested for BAV3-specific IgG response by ELISA (Fig. 8a). At 3 weeks p.i., all the viruses generated similar BAV3-specific IgG antibodies, which were further boosted three- to four-fold following a second inoculation. However, no significant differences were seen among the four groups ($P < 0.5$). The nasal and lung washes collected from BAV3- and mutant-infected cotton rats were tested for BAV3-specific IgA antibodies by ELISA. Cotton rats immunized with BAV429 and BAV430 mutant viruses showed similar IgA titers compared to those animals infected with BAV3 and BAV302 (Fig. 8b).

DISCUSSION

The work described in this article was undertaken primarily to further characterize the BAV3 E4 region. In order to delineate the function of BAV3 E4 gene products in viral replication we constructed a series of mutant

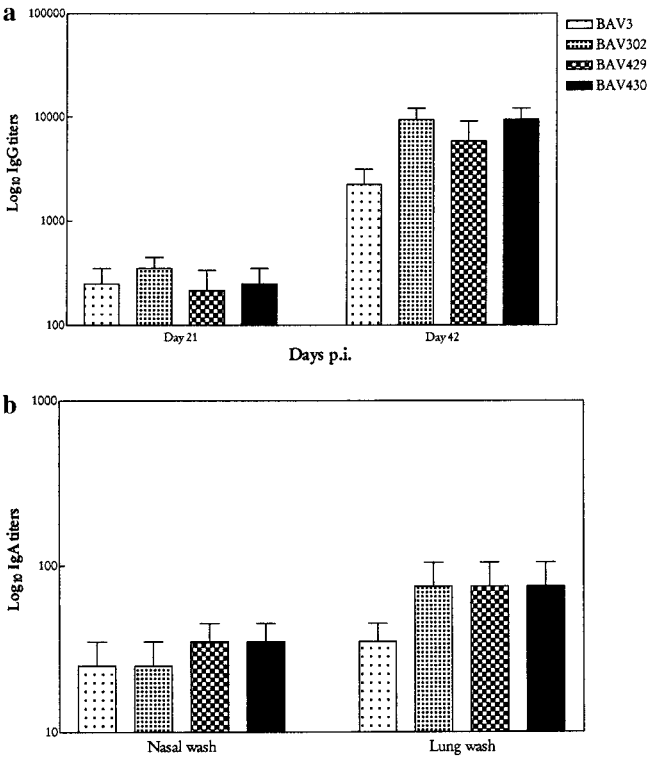


FIG. 8. Antibody response in cotton rats immunized intranasally with BAV3, BAV302, BAV429, and BAV430. (a) BAV3-specific IgG ELISA titers in serum on days 21 and 42 p.i. (b) BAV3-specific IgA ELISA titers in nasal and lung washes on day 42 p.i.

viruses that contain deletions in individual or multiple Orfs of BAV3 E4. Eight mutant viruses were generated following transfection of FBRC cells with DNA containing the various deletions. Studies carried out in the HAV5 E4 region suggest that this region encodes one or more functions which are required for lytic growth in cell culture, as was demonstrated by the defective phenotype of the virus in which the complete E4 was deleted (Halbert *et al.,* 1985; Huang and Hearing, 1989; Krougliak and Graham, 1995; Weinberg and Ketner, 1986). However, HAV5 E4 Orfs 3 and 6 have been shown individually to provide the E4 function necessary for normal viral DNA replication in the absence of other E4 products (Bridge and Ketner, 1989). Thus, E4 Orf3 and Orf6 can partially or totally compensate for each others' deficiencies and the presence of one these Orfs is essential for HAV5 replication. In BAV3 E4, the sequential deletions of the individual Orfs (Orf 1 to 5) suggest that none of the individual Orf appears to be absolutely necessary for virus replication. BAV3 E4 Orf3 and Orf5 show partial homology to HAV5 E4 Orf6; however, simultaneous deletion of Orfs 3 and 5 resulted in the generation of a viable mutant virus, BAV415. This suggests that unlike HAV5 E4 Orf6, BAV3 E4 Orf3/Orf5 do not encode a gene function necessary for viral DNA replication and no functional interaction/compensation exists between the gene products of BAV3 E4 Orfs 3 and 5. This hypothesis was further supported by the successful rescue of mutant virus BAV430, containing deletion of Orf 3 to 5. Also the complete disruption of Orfs 1, 2, and 3 in mutant virus BAV429 suggests that none of the Orfs are essential for virus replication singly or as a complex.

All eight BAV3 E4 mutant viruses were further characterized in terms of growth characteristics, DNA replication, and early/late protein synthesis. None of the E4 mutant viruses appears to be severely defective in their growth, as they were easily propagated on normal MDBK cells. However, based upon their growth characteristics these mutant viruses can be grouped into two classes (I and II; Fig. 4). Mutant viruses in class I have single deletions in individual Orfs from Orf1 to 4 and exhibited growth characteristics similar to those of BAV302. This suggests that single deletions do not have any marked effect on virus propagation. Moreover, disruption of the function of one polypeptide might be substituted by another polypeptide. Mutant viruses in class II either have deletion in Orf5 or contain a larger deletion which disrupts at least three Orfs. These mutant viruses exhibit slower growth characteristics compared to BAV302. The larger deletions and disruption of Orf5 appear to partially inhibit the growth of these recombinant viruses. Similarly, HAV5 E4 mutants have been grouped into three classes (Huang and Hearing, 1989), group III being severely defective for virus growth since they lack both Orf3 and Orf6 gene products. However, no such distinct group was evident in BAV3 E4 mutants that showed severe growth defects. Upon analysis of viral DNA replication, none of mutants carrying a disruption in a single Orf appear to be defective in viral DNA accumulation. However, mutants BAV429 and BAV430, carrying larger deletions, do show a DNA synthesis defect. Both these viruses appear to accumulate lesser amounts of viral DNA at 24, 36, and 48 h compared to BAV302. These observations appear to be consistent with the results obtained in single-step growth curves of these viruses, in which a lag in growth is visible at these time points. However, severe DNA accumulation defects were observed in completely E4-deleted HAV mutants (H2dl808, Weinberg and Ketner, 1986; H2dl366, Halbert *et al.,* 1985).

We next analyzed the viral early ($E1B^{small}$ and DBP) and late (fiber) production of the E4 mutant viruses. There appears to be no severe defect on production of E1 and E2 early proteins by any of the mutant viruses. Some of the BAV3 E4 mutants appear to rather overproduce these early proteins, an observation similar to one seen with HAV E4 mutants that overexpress E2 72K DBP protein (Halbert *et al.,* 1985; Hemstrom *et al.,* 1988). However, deletions in the BAV3 E4 region do appear to partially inhibit fiber production in these mutant viruses with the exception of BAV430, from which the amount of fiber produced is similar to that of BAV302. This observation does support the conclusions drawn from HAV studies (Weinberg and Ketner, 1986; Bridge and Ketner, 1989) that

late protein production is independent of viral DNA accumulation in infected cells.

BAV3 E4 mutants BAV429 and BAV430, which showed a partial defect in virus growth, DNA replication, and early/late protein production *in vitro,* were further characterized *in vivo* by infecting cotton rats intranasally. Both E4 mutant viruses replicated as efficiently was wild-type BAV3 and BAV302 in the lungs of cotton rats, suggesting that these deletions have little effect if any on virus replication *in vivo.* Both deletion mutants were also able to generate similar BAV3-specific IgG and IgA responses in serum and mucosal surfaces of cotton rats compared to BAV3 and BAV302. These results suggest that larger deletions seem to have no effect on mutant BAV429 and BAV430 phenotypes *in vivo,* despite the fact that some phenotypic defects were observed *in vitro.*

Our initial attempts to rescue virus completely devoid of BAV3 E4 by transfecting FBRC cells have not been successful. One of the reasons could be the highly unstable nature of the region in-between BAV3 E4 and the right inverted terminal repeat (ITR). Occasionally, recombinant viruses containing multiple repeat insertions of approximately 500 bp have been identified in-between the TATA box of BAV3 E4 and the right ITR. Similar observations have also been made in the Manhattan strain of CAV2, in which the presence of at least 23 repeats of ~120 to 150 bp in the right ITR resulted in generation of unstable CAV2 virus (Fejer *et al.,* 1992). Another reason might be that complete deletion of BAV3 E4 brings the right ITR in close proximity to the L7 region, which might cause destabilization of the L7 message. The destabilization of the BAV3 L7 region might be due to the spacing between the L7 and the E4 regions, the improper termination of L7 message, or the generation of antisense L7 RNA from the E4 transcripts. Similar studies in HAV showed that when E4 was completely deleted there was a reduction in the accumulation of the late protein fiber (Brough *et al.,* 1996). Further studies are needed to prove or disprove these suggestions.

The primary objective of characterizing BAV3 in greater detail is to use BAV3 for human gene therapy and vaccination of humans and animals. In order to accommodate larger/multiple genes it is necessary to increase the insertion capacity of the BAV3 vector. The insertion capacity of available BAV3 vector is 3.0 kb for replication-competent vectors (deletion in BAV3 E3—1.245-kb deletion; Zakhartchouk *et al.,* 1998) and 3.5 kb for replication-defective vectors (additional E1a region deletion—541 bp, Reddy *et al.,* 1999b), which is sometimes not sufficient to insert larger genes or multiple genes in order to produce multivalent vaccines. The successful deletion of 1501 bp in the E4 region (BAV429) increased the available insertion capacity of replication-competent BAV3 E3–E4 deleted vector and replication-defective BAV3 E1a–E3–E4 deleted vector to ~4.5 and ~5.0 kb, respectively. This will help us to develop safer vaccines in which E1 and E4 have been partially deleted. Moreover, by using the E1, E3, and E4 regions for transgene placement, a single adenovirus can be used for multitransgene expression. This is extremely important when producing cost-effective vaccines, especially in animals, in which multiple antigens can be expressed from a single vector.

In summary, the results of this study suggest that: (i) individual Orfs of the BAV3 E4 can be deleted and thus are nonessential for viral replication; (ii) larger deletions, Orfs 1–3 and Orfs 3–5, also generated viable viruses that grew with slightly slower kinetics compared to E3-deleted virus; (iii) mutant BAV3 virus with the entire E4 region deleted could not be rescued; (iv) *in vivo* characterization of mutant viruses suggests that mutants grow as efficiently as the wild-type BAV3 in the cotton rat model and induce BAV3-specific mucosal and systemic immune response; and, finally, (v) the total available insertion capacity of E3–E4 deleted and E1a–E3–E4 deleted BAV3 vector was increased to ~4.5 and ~5.0 kb.

MATERIALS AND METHODS

Cells and viruses

The WBR-1 strain of BAV-3 was cultivated in MDBK. MDBK and FBRC were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). A purified preparation of BAV3 and mutant viruses was obtained following a double cesium chloride density-gradient centrifugation (Graham and Prevec, 1991). The viral DNA was extracted from virus-infected monolayers by the method of Hirt (1967).

Antibodies

The production and characterization of BAV3 E1B^{small}- and BAV3 DBP-specific antibodies have been described elsewhere (Reddy *et al.,* 1999a; Zhou *et al.,* 2001). The antibodies against E1B^{small} and DBP recognize 19- and 48-kDa proteins, respectively, in BAV3-infected cells. The antibodies generated against the knob domain of BAV3 fiber recognize a protein of 102 kDa in BAV3-infected cells (Wu *et al.,* manuscript in preparation).

Construction of recombinant plasmids

The recombinant plasmid vectors were constructed by standard procedures (Sambrook and Russell, 2001), using restriction enzymes and other DNA-modifying enzymes as described by the manufacturers. In order to create deletions in the BAV3 E4 region, transfer plasmid E4polyIISn, described elsewhere (Baxi *et al.,* 1999), was used. A schematic representation of various E4 deletion mutants is described in detail in Fig. 1. The deletion in E4 Orf1 was generated by digesting E4polyIISn with *Sca*I–*Mlu*I (33905–33762 bp on BAV3 genome) and inserting an *Eco*RV linker. E4 Orf2 was deleted by digesting

E4polyIISn with *Bcl*I–*Afl*II (33305–33247 bp) and inserting a *Srf*I linker. A *Pst*I–*Hpa*I fragment (32576–32404 bp) was deleted from the E4 Orf3 and a *Srf*I linker was inserted. E4 Orf4 was deleted by digesting with *Sp*I–*Ppu*MI (31744–317606 bp) and inserting a *Srf*I linker. E4 Orf5 carries a *Nco*I–*Xma*I (31680–31530 bp) deletion and has a *Srf*I linker inserted in the deletion. In order to generate Orf1 to 3 deletion, *Sca*I–*Hpa*I (33905–32404 bp) was deleted and a *Srf*I linker was inserted. Digestion of E4polyIISn with *Pst*I–*Dra*III (32576–31234 bp) created an Orf3 to 5 deletion in E4 region and a *Srf*I linker was inserted. Full-length plasmids having various deletions in the E4 region were generated by using the homologous machinery of *Escherichia coli* (Chartier *et al.,* 1996). Transfer plasmids having the deletions in E4polyIISn were digested with *Eco*RI–*Ahd*I and homologous recombination was carried out with *Swa*I-linearized pFBAV302 in *E. coli* BJ5183 cells to generate the full-length plasmids.

Transfection of cells

FBRC cell monolayers in six-well plates were transfected with 2 to 5 μg of various *Pac*I-digested recombinant full-length plasmid DNAs using Lipofectin (Gibco BRL). Following transfection, cells were maintained in MEM containing 2% FBS at 37°C for 15–20 days until cytopathic effects appeared. Cells showing 50% cytopathic effects were harvested and freeze–thawed three times, and recombinant virus was plaque-purified on MDBK cells.

Polymerase chain reaction

PCR was carried out to verify the deletions created in the E4 mutant viruses. MDBK cells were infected with the various mutant viruses and viral DNA was extracted according to the method of Hirt (1967). Amplification products were generated by creating primers in the 5′ and 3′ flanking regions of the deletion (details of the primers used are shown in Table 1). The PCR mix contained 20 pmol of each primer, 0.2 mM dNTPs, 1.5 U *Taq* polymerase, 1 × reaction buffer, and the template viral DNA. The PCR was of a standard design with 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 2 min. This was preceded by an initial denaturing step of 94°C for 5 min and completed by a final extension step of 72°C for 10 min. The amplificates were analyzed by electrophoresis in a 2% agarose gel and visualized with ethidium bromide.

Immunoprecipitations

Monolayers of MDBK cells were infected at m.o.i. of 10 of BAV302 or mutant viruses. After 90 min of absorption, cells were washed and incubated in MEM containing 2% FBS. At 24 h.p.i., the cells were incubated in glucose-free Dulbuco's modified Eagle's medium for 1 h before being labeled with [^{35}S]methionine (100 μCi/ml). After 12 h of labeling, cells were washed with PBS and harvested in radioimmunoprecipitation buffer [RIPA buffer: 150 mM NaCl, 10 mM Tris (pH 7.8), 1% Triton X, 1% sodium deoxycholate, 0.1% SDS]. Immunoprecipitations were carried out with polyclonal antibodies against BAV3 E1B^{small}, DNA binding protein, and fiber, and complexes were captured with protein A–Sepharose beads. Precipitates were washed in RIPA buffer and proteins were separated by 10% SDS–PAGE. Labeled proteins were visualized by autoradiography.

Viral DNA dot-blot assay

In order to analyze DNA accumulation, MDBK cells were infected with BAV302 or mutant viruses at m.o.i. of 10 and total viral DNA was isolated at 24, 36, and 48 h after infection. The DNA was denatured in 0.3 N NaOH at 65°C, neutralized with 2 M ammonium acetate (pH 7.0), diluted 1:10 and 1:100, and transferred onto nitrocellulose by using a dot-blot apparatus. The blots were then probed by Southern blot hybridization analysis, using random-primed ^{32}P-labeled BAV3 DNA. The results were visualized by autoradiograph.

Animal experiments

A total of 36 6- to 8-week-old cotton rats were divided into four groups (nine animals/group) and inoculated intranasally with 10^7 pfu of purified virus/animal. The four groups of cotton rats were infected with BAV3, BAV302, BAV429, or BAV430. At 2, 3, and 6 days postinoculation, animals were sacrificed with an overdose of halothane and the right lungs from inoculated animals were collected in MEM for virus isolation. The left lung was inflated with and fixed in 10% neutral-buffered formalin for histopathogical analysis according to the protocol standardized by Mittal *et al.* (1995).

In the second experimental group, 12 cotton rats were infected intranasally with 10^7 pfu purified virus/animal at 0 and 3 weeks p.i. The cotton rats were divided into four groups and were infected with BAV3, BAV302, BAV429, or BAV430. At 3 and 6 weeks p.i. blood samples were collected to monitor the development of BAV3-specific antibodies in ELISA and virus neutralization assay. Nasal and lung washes were also collected at 6 weeks p.i. to determine mucosal antibody responses against BAV3.

Virus isolation

The right lungs were collected from cotton rats inoculated with various viruses and stored at −70°C until use. Tissues were weighed, homogenized in 1 ml of MEM (Polytron homogenizer; Brinkmann Industries, Rexadle, Canada), and centrifuged at 3000 rpm to remove the debris. The resulting supernatant was used for virus titration on MDBK cells and results were expressed as $TCID_{50}$/ml.

BAV3-specific ELISA

The levels of BAV3-specific antibodies in sera, lung, and nasal washes were determined by ELISA. Immunolon-2 microtiter plates were coated with purified BAV3 (0.1 μg/well) in coating buffer at 4°C overnight. The plates were washed with PBST between each step. All samples were serially diluted in PBST-BSA and incubated overnight at 4°C. Antigen-specific IgG was detected by horseradish peroxidase-conjugated goat anti-rat IgG (Cedarlane). Antigen-specific IgA was measured by rabbit anti-rat IgA, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG. Reactions were visualized with streptavidin–alkaline phosphate conjugate and BCIP/NBT.

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